

Immunodominant Regions within the Hepatitis C Virus Core and Putative Matrix Proteins

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The complete amino acid sequences of hepatitis C virus (HCV) core (residues 1 to 115) and putative matrix (residues 116 to 190) proteins were synthesized as 18-residue-long peptides with an 8-amino-acid overlap. The peptides were assayed with 50 human serum samples with antibodies to HCV (anti-HCV) and 46 serum samples without anti-HCV, as determined by several commercial assays. Immunodominant regions were defined within residues 1 to 18, 11 to 28, 21 to 38, 51 to 68, and 101 to 118. The peptides that covered these regions were recognized by 40 of 50 (80%), 42 of 50 (84%), 36 of 50 (72%), 34 of 48 (68%), and 36 of 48 (72%) of the anti-HCV positive serum samples, respectively. Two anti-HCV negative serum samples were each repeatedly reactive with one peptide, but both were found to be negative by confirmatory anti-HCV assays. Four serum samples that were confirmed to be positive for anti-HCV in commercial assays did not recognize any of the peptides that cover the HCV core-matrix regions. Ninety-two percent of anti-HCV-positive serum samples reacted with a combination of peptides covering residues 1 to 18 and 11 to 28. Testing of peptides that contain the reported genotypic variations of the HCV core within the regions at residues 1 to 18, 51 to 68, and 101 to 118 showed that a change from Thr-110 to Asn-110 decreased the reactivities of eight serum samples. In conclusion, we found that human antibodies to the HCV core-matrix protein(s) are mainly directed to linear determinants and can easily be reproduced by using short synthetic peptides. We also found that such antibodies develop in more than 90% of HCV-infected people.

The hepatitis C virus (HCV), which was first described in 1989 (4, 12), is a member of the flaviviridae (5, 11, 19, 27). The genome appears to encode for three structural proteins, the core, putative matrix, and envelope proteins, and possibly six nonstructural (NS) proteins, NS1, NS2, NS3, NS4a, NS4b, and NS5 (23). HCV is prevalent at rates of between <0.36 and 1.0% among blood donors in industrialized countries (6, 10, 13, 20) and at rates of about 2.5 to 6.4% among populations in developing countries (1, 26). At present, the most commonly used assays in Europe and the United States use the recombinant C-100-3 construct that covers parts of the NS4 protein (4), although recently, new assays that contain either long synthetic peptides or recombinant peptides that cover both structural and nonstructural HCV products have been introduced (8). The problems with the early assays have been unsatisfactory sensitivities and specificities (6), although the new assays seem to have both improved sensitivities and specificities, and therefore, the accuracies of the assays are improved (2). We were interested in characterizing further the immune recognition of HCV and to identify the immunodominant regions within the HCV core protein by using short synthetic peptides.

MATERIALS AND METHODS

Patient sera. A total of 96 serum samples were selected from consecutive serum samples sent to the National Bacteriological Laboratory from March 1991 to May 1991. They were selected because they had been assayed for anti-HCV by commercial assays (first- and second-generation tests [Abbott Laboratories, North Chicago, Ill.], Heganostica C [Organon Teknika, Boxtel, The Netherlands], or second-generation tests [Ortho Diagnostic Systems, Raritan, N.J.]).

On testing, 50 of the serum samples were found to be reactive and 46 were found to be negative by the commercial assays. The majority (40 of 50) of the anti-HCV-positive sera were obtained from intravenous drug users.

HCV core and putative matrix amino acid sequence. The amino acid sequences of the HCV core (residues 1 to 115)

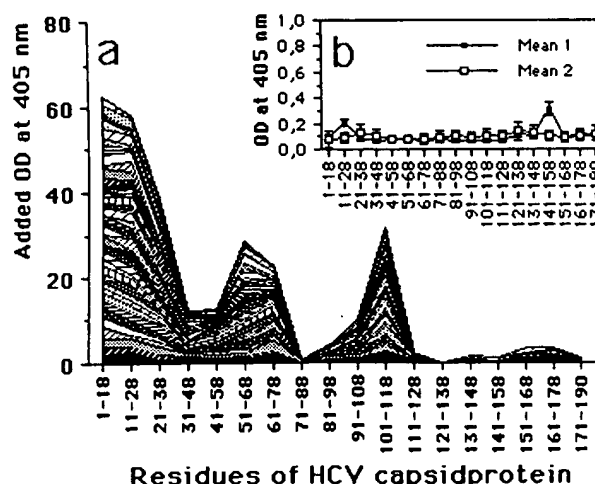


FIG. 1. Sum of OD₄₀₅ values for positive reactions with 44 anti-HCV-positive sera (a). Only sera that gave an OD₄₀₅ that exceeded the mean \pm 7 standard deviations for negative sera are included. (b) Mean \pm standard deviation OD₄₀₅ for negative sera for the peptides covering the HCV core and matrix proteins; data are from two different runs; mean 1 (n = 46 serum samples); mean 2 (n = 12 serum samples).

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TABLE 1. Epitope mapping of 18-amino-acid peptides with an 8-amino-acid overlap with 50 human serum samples found to be positive for anti-HCV by C-100-3 or multiple antigen assays^a

Serum sample	Strength of reactivity with the indicated 18-amino-acid peptide starting at residue ^b :																	
	1	11	21	31	41	51	61	71	81	91	101	111	121	131	141	151	161	171
2382	+++	+++	+++	+	+	+	+++	+	+	+	++	+				+	+	
2823	+++	+++	++	++	++	++	+++		+	++	+++	+		+		+	+	+
4952	++	++	+	+	+	++	++		+	+	++	+				+	+	
2435	+++	+++	++	+	+	++	++		+	++	++					++	+	+
3850	+++	+++	++	+	+	++	++			+	++	+				+	+	
2271	++	+	+	+	+	+	++			+	++				+			
3818	+++	+++	++	+	+	++	+			+	+	+						
3710	++	++	++	+	+	++	+			+								
2342	+++	++	+	+		+	++				++							
2840	+++	++	+	+		+	+				+							
3754	+++	+	+++	+		+					+							
3842	++	+	+	+		+					+					+		
3596	++	++	++	+							+							
3897	++	++	+			+	+			+	++			+				
2089	++	++	+			+	+				++					+		
2311	+	++	++			+	+				+							
3867	+++	+++	++			+	+				+							
3830	+++	++	++			+					+							
3855	+++	+	++			+					+							
2426	++	++	++			+									+			
3553	+	++	+						+		+					+		+
3765	+	+++	++								++							
3891	++	+	++								+							
2090	+	+++	+		++	++	++		+	+	+						+	
2276	++	++	++		++	++	+			+	+							
2272	++	+++	+		+	++	+			+	++							
2457	+++	++	+		+	+	+											
5074	+++	++	++		+	+												
2378	+++	+	++		+	+	+				+							
3563	++	+	+			+												
2269	++	++		+	+	++	++			+	+							
5096	+	++		+		++	+				+							
3734	++	++				+++	+			+	+							
3764	+++	++			+	++	+			+	+							
3601	+	++			+	+					+							
3865	++	++			+									+				
5087	+++		++	++		+	+											
2264	++		+								+							
3743	++			+														
3893	+					+												
5086		+++	+		+	+	+		+	+	+++						+	
3554		++	+		+						++							
3599		+	++	++							+					+		+
5068		++	+	+							++							
3750		++				+					+							
3733		+									+							
3611																		
3828																		
3873																		
5070																		
Total no. of serum samples that were reactive	40	42	36	20	20	34	25	1	7	16	36	5	0	3	2	9	7	4

^a The cutoff was set at the means \pm 7 standard deviations for negative serum samples. Sera were sorted according to the recognition pattern of the N-terminal portion at residues 1 to 58 only to increase readability.

^b The core protein was from residues 1 to 115; the matrix protein was from residues 116 to 190. +, OD₄₀₅ of <1.0; ++, OD₄₀₅ of >1.0; +++, OD₄₀₅ of >2.0; OD₄₀₅ values were determined by peptide enzyme immunoassay.

TABLE 2. Reactivities of combinations of peptides from three immunodominant regions within the HCV core in detecting 50 anti-HCV-positive sera^a

HCV core peptide combination (residues)	No. (%) of sera reactive	OD ₄₀₅ (range) of reactive sera	OD ₄₀₅ (mean \pm SD) of anti-HCV-negative sera
1-18 and 11-28	46 (92)	0.548-2.600	0.087 \pm 0.019
1-18 and 51-68	44 (88)	0.220-2.600	0.074 \pm 0.016

^a OD values that exceeded the mean \pm 7 standard deviations were regarded as reactive. The peptide combinations were assayed at various concentrations (0.01 to 0.001 mg/ml), and the concentrations that gave the highest sensitivity were used to obtain the results presented here.

and the putative matrix (residues 116 to 190) proteins were obtained by comparing previously sequenced genomes (5, 11, 18, 19, 23-25). The sequence from a U.S. strain (24) was selected and synthesized as 18-residue peptides with an 8-residue overlap between each peptide.

Also, a set of five of peptides within the immunodominant regions that are reported to vary among strains (residues 1 to 18, 51 to 68, and 101 to 118) (5, 11, 18, 19, 23, 24) was synthesized. The peptides were as follows: one peptide with an Ile-4 instead of an Asn-4, one peptide with a Lys-6 instead of an Arg-6 and an Asn-11 instead of a Thr-11, one peptide with a Trp-61 instead of an Arg-61, one peptide with a Val-68 instead of an Ala-68, and one peptide with an Asn-110 instead of a Thr-110.

A total of 23 different HCV core peptide analogs were synthesized, and all except the 5 analogs containing strain-specific amino acid substitutions were assayed by using the 96 serum samples.

Peptide synthesis. Peptides were simultaneously synthesized by a slightly modified version of a recently described method (21). In brief, the peptides were synthesized on 30 mg of Polyhipe resin (NovaSyn PR 500; NovaBiochem, L  ufingen, Switzerland) held in polypropylene bags. All amino acids were prepared in stock solutions and were dispensed to the appropriate peptide bag at each coupling step. Throughout the synthesis, *N,N*-dimethylformamide was used as the sole solvent for the coupling and washing steps. The final peptides were then cleaved and deprotected

by using trifluoroacetic acid containing the appropriate scavengers (21). All peptides were analyzed by high-pressure liquid chromatography. Peptides were lyophilized and then dissolved in distilled water at a concentration of 1 mg/ml.

Peptide enzyme immunoassays. Peptides were coated onto microtiter plates (Maxisorp 96F Certificate; Nunc, Roskilde, Denmark) at 0.001 mg per well overnight at 4°C in NaHCO₃ (pH 9.6). Before testing, the plates were blocked by the addition of 2% bovine serum albumin for 2 h at room temperature. Sera were then added at a 1:100 dilution and were incubated for 45 min at 37°C. Serum-bound immunoglobulin G was then detected by adding alkaline phosphatase-labeled goat anti-human immunoglobulin G (A-3150; Sigma Chemical Co., St. Louis, Mo.) diluted 1:1,500.

Dilutions of sera and conjugate were prepared in phosphate-buffered saline containing 2% goat serum, 2% bovine serum albumin, and 0.05% Tween 20.

In inhibition experiments, the enzyme immunoassay was performed as described above by adding 0.01 to 0.03 mg of the respective peptide at the same time that sera were added to the coated wells.

Since unspecific reactions have been one of the major problems with commercial anti-HCV assays, we wanted to avoid this in our assays, and therefore, to obtain a high specificity, only reactions with absorbances that exceeded the means \pm 7 standard deviations of the absorbances of anti-HCV-negative sera were regarded as reactive.

Hydropathic index of the HCV core-matrix protein sequence. The hydropathic index of the HCV core-matrix protein sequence (residues 1 to 190) was predicted and plotted by the method described by Kyte and Dolittle (14) by using Micro Genie software on a personal computer (International Business Machines).

RESULTS

Mapping of antigenic regions within HCV core-matrix proteins. Results from the testing of 50 anti-HCV positive serum samples with the 18 HCV core-matrix peptides are given in Fig. 1a and Table 1, and the mean optical density at 405 nm (OD₄₀₅) of the anti-HCV negative sera is given in Fig. 1b. The values in Fig. 1a are given as the cumulated absorbances (sums of the OD₄₀₅s) of all positive reactions for

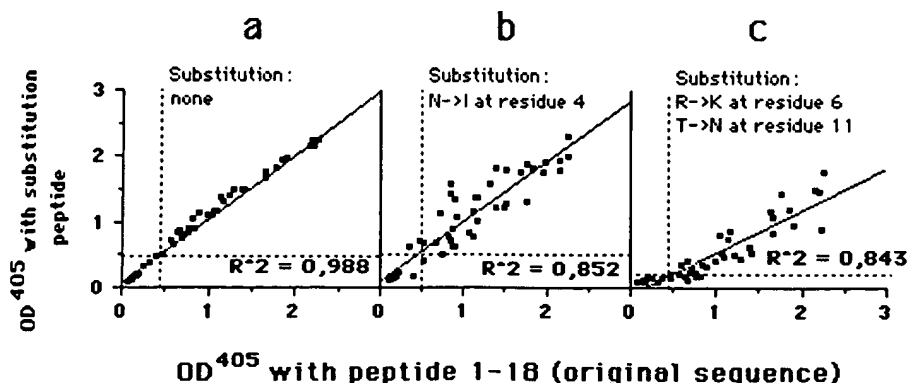


FIG. 2. Comparison of two runs with 44 anti-HCV-positive and 4 anti-HCV-negative sera with the original peptide at residues 1 to 18 (a). The same serum samples were used to compare the recognition of the original peptide at residues 1 to 18 with the recognition of two peptides covering residues 1 to 18 containing the strain-specific substitutions Asn-4 to Ile-4 (N to I) (b) and Arg-6 to Lys-6 and Thr-11 to Asn-11 (R to K, and T to N, respectively) (c). Values are given as the OD₄₀₅, and correlation is given as R² values.

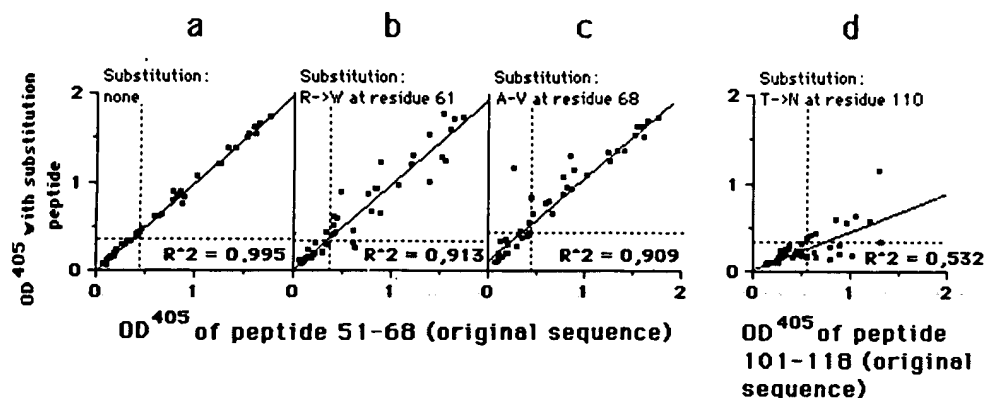


FIG. 3. Comparison of two runs with 44 anti-HCV-positive and 4 anti-HCV-negative sera with the original peptide at residues 51 to 68 (a). The same sera were used to compare the recognition of the original peptides at residues 51 to 68 and 101 to 118 with the recognition of three peptides covering residues 51 to 68 and 101 to 118 containing the strain-specific substitutions Arg-61 to Trp-61 (R to W) (b), Ala-68 to Val-68 (A to V) (c), and Thr-110 to Asn-110 (T to N) (d). Values are given as the OD₄₀₅, and correlation is given as R² values.

each peptide obtained with the sera that were positive by commercial anti-HCV assays. The major antigenic regions were covered by peptides 1 to 18, 11 to 28, 21 to 38, 51 to 68, and 101 to 118. The regions at residues 1 to 18, 11 to 28, and 21 to 38 seemed to contain distinct antigenic regions, since some sera were found to react with each of these peptides and not with the adjacent overlapping peptides (Table 1; for example, sera 5087, 3750, and 3733). Peptides 51 to 68 and 61 to 68 were also found to have reactivities, suggesting two distinct sites. Most serum samples showed combined reactivities to regions 1 to 38, 51 to 68, and 101 to 118; only two serum samples recognized fewer than three peptides. The peptide reactivities of the anti-HCV-positive sera could be inhibited by more than 50% by adding the specific peptide, suggesting specific reactions.

None of the 18 HCV core-matrix peptides was detected by all 50 anti-HCV-positive serum samples. Most sera reacted with the peptides at residues 1 to 18 and 11 to 28, which were detected by 40 and 42 serum samples, respectively. Thirty-six serum samples were detected by the peptide at residues 21 to 38, 34 serum samples were detected by the peptide at residues 51 to 68, and 36 serum samples were detected by the peptide at residues 101 to 118. The negative sera had a higher background reaction when they were initially tested with the peptides at residues 11 to 28 and 141 to 158 (Fig. 1b). Upon retesting reactivities were found to be reduced to a level similar to those of the other peptides.

Of the 46 anti-HCV negative serum samples, 2 serum samples were each found to react reproducibly with one peptide of the HCV core peptides. These two serum samples were found to be reproducibly negative by using several second-generation anti-HCV assays, even though the peptide reactivities of both serum samples to the HCV core could be inhibited by adding the specific peptide to the solution. These reactions were not regarded as indicative of previous exposure to HCV. Four of the 50 anti-HCV-positive serum samples were not reactive with any of the HCV core-matrix peptides. All four were found to react with nonstructural recombinant proteins in the Supplemental Assay (Abbott); and by the same assay and with the structural recombinant proteins, one gave strong reactivity, two gave values just above the cutoff, and one was nonreactive.

Testing of a combination of HCV core-matrix peptides. A

combination of peptides covering residues 1 to 18 and 11 to 28 for coating showed a complementary function and increased the sensitivity to 92% (Table 2). The inclusion of the peptide at residues 51 to 68 did not add to the sensitivity or to the positive-to-negative ratio when assayed with our sera.

Testing of genotype-specific HCV core-matrix peptides. Testing of the genotype-specific peptides showed that sera with low-level reactivities (OD₄₀₅ < 0.5) almost exclusively changed their reaction patterns when they were assayed with these peptides (Fig. 2 and 3). The reactivity to the N-terminal portion of the HCV core protein showed a stable cross-reaction between peptides from HCV strains of different genotypes (Fig. 2).

One serum sample became reactive to the peptide at residues 51 to 68 if residue Ala-68 was substituted by Val-68 (Fig. 3c). The amino acid-specific change from Thr-110 to Asn-110 was found to be the most sensitive substitution (Fig. 3d), and eight reactive serum samples became negative. The reactivities recorded with the substitution peptides were significantly correlated to the reactivities with the original peptide ($P < 0.001$ for all; regression analysis). Also, the reproducibilities of the peptide assays were found to be high when we compared duplicate samples run on different occasions (Fig. 2a, $P < 0.001$, and Fig. 3a, $P < 0.001$; regression analysis).

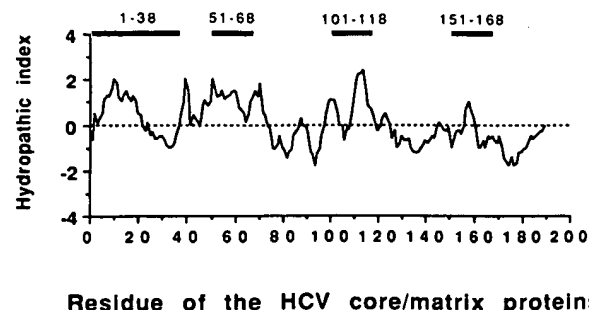


FIG. 4. Immunodominant regions within the HCV core and matrix proteins with respect to the hydropathic character plotted by the method by Kyte and Doolittle (14).

The hydrophobic character of the HCV core-matrix peptide sequence. The hydrophobic character of the HCV core-matrix peptide amino acid sequence (14) is plotted in Fig. 4 in relation to the immunodominant regions. All regions contained hydrophilic peaks.

DISCUSSION

Using short synthetic peptides, we identified three immunodominant regions within the HCV core protein, all of which were hydrophilic. Our data suggest that the major region at residues 1 to 38 contains a minimum of three distinct binding sites, whereas the region at residues 51 to 78 might contain two distinct binding sites, and the region at residues 101 to 118 contains only one distinct binding site. The putative matrix protein or carboxy-terminal half of the core protein seems to contain fewer immunogenic regions, with the major linear site located within residues 151 to 168. The immunodominant regions within the HCV core protein are easily reproduced by short synthetic peptides, suggesting that the epitope regions contain linear determinants. This suggestion corroborates the finding of Nasoff et al. (17), who were able to detect reactivities to the HCV core protein by using short recombinant constructs of the N-terminal portion of the HCV core peptide. This is different from what has been shown for some other viral proteins, such as the hepatitis B virus core antigen, in which the main recognition sites have been shown to be discontinuous (7, 20).

Four of the 48 initially anti-HCV-negative serum samples showed reproducible and inhibitable reactivities to some of the HCV core peptides, and at a later stage, two of the serum samples were confirmed to be anti-HCV positive. The other two serum samples with a low level of reactivity to single peptides were likely unspecific.

The amino acid variations within the identified immunodominant regions did not have a large impact on immunological recognition, and therefore, it can be assumed that antibodies to determinants within the HCV core are mainly cross-reactive between the various strains of HCV.

The combination of two HCV core peptides gave a sensitivity of 92% in detecting anti-HCV-positive sera, as determined by a combination of anti-HCV assays containing recombinant structural proteins, nonstructural proteins, or both. In two other series of serum samples, one series from blood donors and one series from immunosuppressed patients, we isolated seroreactivities to single HCV core peptides covering the regions at residues 21 to 38, 31 to 48, and 51 to 68, which suggests an increased sensitivity when these peptides are included (unpublished data).

The inclusion of the HCV core peptide in new commercial kits was a necessary step, since studies have shown that the assay with the recombinant C-100-3 gives a high rate of nonspecific reactions in blood donor populations (16) and also that anti-C-100-3-negative donors may transmit HCV (9). Also, inclusion of the core and NS3 proteins shortens the time for seroconversion in the serodiagnosis of HCV (15).

However, we found that about 4 to 8% of HCV-infected people do not have antibodies to the HCV core protein. This is similar to the results of Chiba et al. (3), who found that 5.2% of the 58 serum samples from persons with clinically well defined chronic non-A, non-B viral hepatitis did not react with a recombinant core protein.

In conclusion, we showed that short synthetic peptides mimic the antigenic regions found on the HCV core protein. Thus, the defined immunodominant regions should be further evaluated regarding their potential as tools in character-

izing the immune response to the HCV core protein during infection.

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Sequence analysis of the core gene of 14 hepatitis C virus genotypes

(non-A, non-B hepatitis/genetic heterogeneity/polymerase chain reaction/phylogenetic tree/taxonomy)

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ABSTRACT We previously sequenced the 5' noncoding region of 44 isolates of hepatitis C virus (HCV), as well as the envelope 1 (E1) gene of 51 HCV isolates, and provided evidence for the existence of at least 6 major genetic groups consisting of at least 12 minor genotypes of HCV (i.e., genotypes I/1a, II/1b, III/2a, IV/2b, 2c, V/3a, 4a–4d, 5a, and 6a). We now report the complete nucleotide sequence of the putative core (C) gene of 52 HCV isolates that represent all of these 12 genotypes as well as two additional genotypes provisionally designated 4e and 4f that we identified in this study. The phylogenetic analysis of the C gene sequences was in agreement with that of the E1 gene sequences. A major division in the genetic distance was observed between HCV isolates of genotype 2 and those of the other genotypes in analysis of both the E1 and C genes. The C gene sequences of 9 genotypes have not been reported previously (i.e., genotypes 2c, 4a–4f, 5a, and 6a). Our analysis indicates that the C gene-based methods currently used to determine the HCV genotype, such as PCR with genotype-specific primers, should be revised in light of these data. We found that the predicted C gene was exactly 573 nt long in all 52 HCV isolates, with an N-terminal start codon and no in-frame stop codons. The nucleotide and predicted amino acid identities of the C gene sequences were in the range of 79.4–99.0% and 85.3–100%, respectively. Furthermore, we mapped universally conserved, as well as genotype-specific, nucleotide and deduced amino acid sequences of the C gene. The predicted C proteins of the different HCV genotypes shared the following features: (i) high content of proline residues, (ii) high content of arginine and lysine residues located primarily in three domains with 10 such residues invariant at positions 39–62, (iii) a cluster of 5 conserved tryptophan residues, (iv) two nuclear localization signals and a DNA-binding motif, (v) a potential phosphorylation site with a serine-proline motif, and (vi) three conserved hydrophilic domains that have been shown by others to contain immunogenic epitopes. Thus, we have extended analysis of the predicted C protein of HCV to all of the recognized genotypes, confirmed the existence of highly conserved regions of this important structural protein, and demonstrated that the genetic relatedness of HCV isolates is equivalent when analyzing the most conserved (i.e., C) and the most variable (i.e., E1) genes of the HCV genome.

Hepatitis C virus (HCV) is an important human pathogen that can cause acute and chronic hepatitis, liver cirrhosis, and, possibly, hepatocellular carcinoma. The virus particles contain a positive polarity, single-stranded RNA genome with 5' and 3' noncoding (NC) regions. The core (C), envelope 1 (E1), and envelope 2 proteins are encoded at the 5' terminus and the nonstructural proteins are encoded at the 3' terminus of the single open reading frame of the genome (1, 2). It is now

well established that there are a number of different genotypes of HCV, which may have important implications for pathogenesis, diagnosis, and vaccine development. Based on analysis of HCV isolates sequenced in their entirety, Okamoto *et al.* (3) demonstrated that all previously published sequences could be grouped into four such genotypes [i.e., genotypes I/1a (2), II/1b (4), III/2a (5), and IV/2b (3)]. At approximately the same time, our analysis of the highly conserved 5' NC region of 44 HCV isolates from around the world suggested the existence of additional genetic groups of HCV (6). Our subsequent analysis of the highly variable E1 gene from 51 HCV isolates (7) confirmed the presence of 12 genotypes divided into at least six major genetic groups and accompanying subgroups (i.e., genotypes I/1a, II/1b, III/2a, IV/2b, 2c, V/3a, 4a–4d, 5a, and 6a). Genotype V/3a isolates have also been found by others (8–12). Subsequently, Simmonds *et al.* (13) confirmed the existence of multiple major genetic groups of HCV by sequence analysis of a short region within the NS-5 gene. However, until definitive overlapping sequences are available, the genetic relatedness of HCV isolates designated genotypes 4, 5, and 6 by Simmonds and coworkers to our isolates of genotypes 4a–4d, 5a, and 6a cannot be determined. Taken together, it is clear that there are at least 12 genotypes of HCV.

In this report, we have designated the various genotypes by the nomenclature proposed by Okamoto *et al.* (3) and by Chan *et al.* (9). However, as we have pointed out (7), the proposed classification schemes should be considered provisional until more data are obtained. In the present study, we have determined the complete nucleotide sequence of the C gene in 52 HCV isolates* that represent the 12 recognized HCV genotypes as well as two additional genotypes, 4e and 4f, identified in this study.

MATERIALS AND METHODS

Sera analyzed in this study were from 52 individuals from 12 countries, who were positive for antibodies to HCV (anti-HCV) by a first-generation test (14). The consensus 5' NC and E1 gene sequences of the HCV RNA from these sera were previously analyzed (6, 7). In this study we have analyzed the consensus C gene sequence of these same HCV isolates. The procedures that were used for viral RNA extraction, cDNA synthesis, and nested PCR have been described (14). For the cDNA PCR assay, we used HCV-specific synthetic oligonucleotides deduced from previously determined sequences that flank the C gene (6, 7). In 51 of the 52 HCV isolates studied, we amplified the entire C gene and adjacent 5' NC and E1 sequences. However, in isolate Z7 the 36 nt at the 3' end of C were from a second DNA fragment that we obtained previ-

Abbreviations: HCV, hepatitis C virus; NC, noncoding; C, core; E1, envelope 1.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U10189–U10240).

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ously (7). Amplified DNA was purified by gel electrophoresis followed by glass-milk extraction (7) or electroelution and both strands were sequenced directly. In 44 of the 52 HCV isolates studied, we used the procedures for direct sequencing described previously (7). For a number of the HCV isolates, confirmatory sequencing was performed with the Applied Biosystems automated DNA sequencer (model 373A) and 8 HCV isolates of genotype I/1a or II/1b were sequenced exclusively by this method. Multiple sequence alignments were performed with the program GENALIGN (15). Phylogenetic trees were constructed by the unweighted pair-group method with arithmetic mean that is based on the assumption of a constant rate of evolution (16).

RESULTS AND DISCUSSION

In this study, we successfully reverse-transcribed and, by PCR, amplified the entire C gene from HCV isolates representing the 12 genotypes identified by analysis of E1 sequences (7) and from all of the HCV isolates with unique 5' NC sequences (6) that we could not amplify in the previous study of E1. All 73 negative control samples interspersed among the test samples were negative for HCV RNA. The amplified DNA fragment obtained in 50 of the 52 HCV isolates was specifically designed to overlap with our previously obtained 5' NC-C sequences (6) and C-E1 sequences (7) at ≈ 80 nt positions each. A complete match was observed in 6033 of 6035 overlapping nt. Two discrepancies were observed in isolate US6 at nt 552 (C and T) and 561 (C and T). This may have been due to microheterogeneity at these

nucleotide positions, since the remaining overlapping sequence was unique for isolate US6. In addition, there were three confirmed instances of microheterogeneity: nt 33 in isolate SA11 (C, T, and T), nt 36 in isolate S45 (A, C, and A), and nt 552 in isolate P10 (C, T, and T). Overall, the excellent agreement in these overlapping sequences in this study with that of the two previous studies definitively ruled out contamination as a source of nonauthentic HCV sequences. Furthermore, this analysis proved that the sequences obtained were from a single population and not from different populations as could happen in mixed infections.

Analysis of the Nucleotide Sequence of the C Gene. We now report the nucleotide (nt 1–573) and deduced amino acid (aa 1–191) sequences of the putative C gene of 52 HCV isolates. Relative to the prototype sequence (1, 2), we found that the C gene was exactly 573 nt long in all 52 HCV isolates with an N-terminal start codon and no in-frame stop codons. Microheterogeneity, defined previously (7), was observed in 26 of the 52 HCV isolates at 0.2–1.4% of the 573 nucleotide positions of the C gene and resulted in changes in 0.5–1.0% of the 191 predicted aa in 12 of these isolates. We performed a multiple sequence alignment (data not shown) and found that the nucleotide identities of the C gene among these HCV isolates were in the range 79.4–99.0%. Since we were interested in comparing the genetic relatedness of HCV isolates in different gene regions we constructed phylogenetic trees of the C gene of all 52 HCV isolates from this study and the E1 gene of 51 HCV isolates from our previous study (7) using the unweighted pair-group method with arithmetic mean (16) (Fig. 1). In both dendrograms we observed a division of the

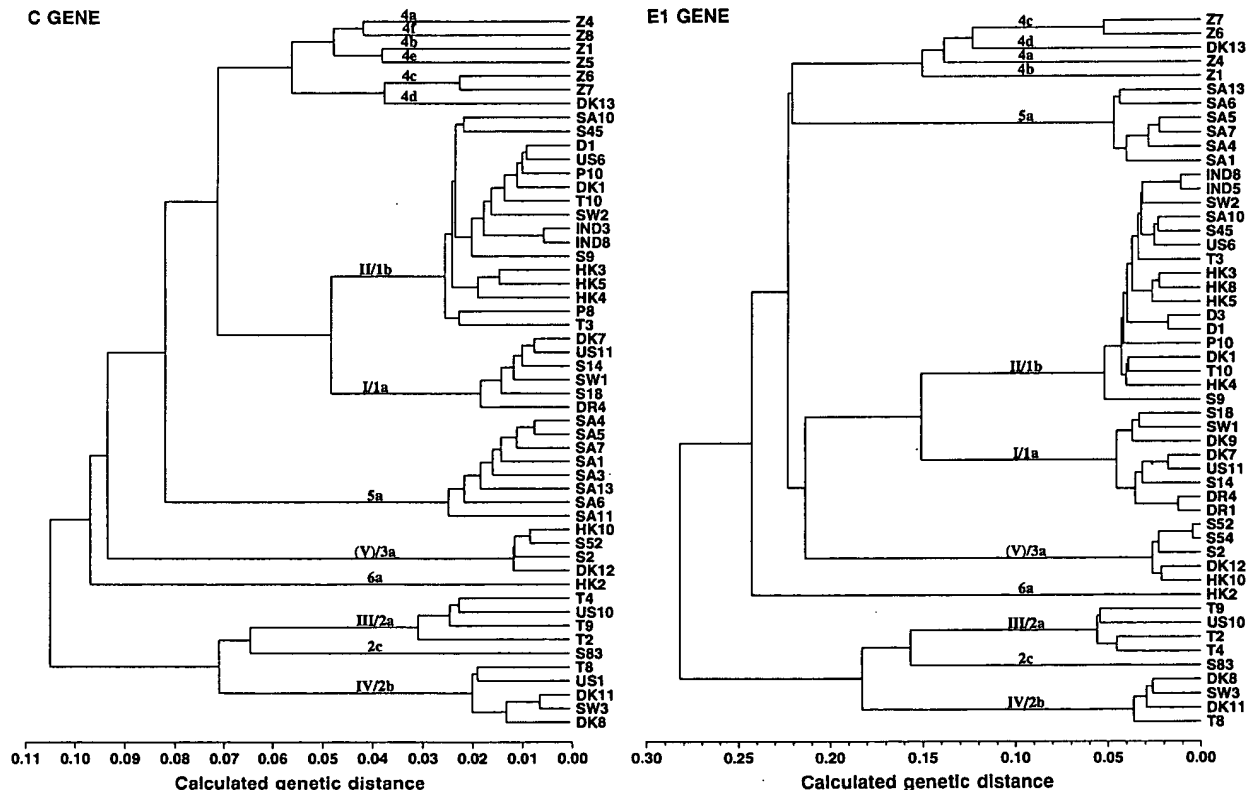


FIG. 1. Phylogenetic trees showing calculated evolutionary relationships of the different HCV isolates based on the C gene sequence of 52 HCV isolates and the E1 gene sequence of 51 HCV isolates. Phylogenetic trees were constructed by the unweighted pair-group method with arithmetic mean (16) using the computer software package GENWORKS from IntelliGenetics. Lengths of the horizontal lines connecting the sequences, given in absolute values from 0 to 1, are proportional to the estimated genetic distances between the sequences. Genotype designations of HCV isolates are indicated. In 45 HCV isolates, we determined both the C and the E1 gene sequences.

45 HCV isolates that were common to the two studies into at least six major genetic groups (genotypes 1–6) and 12 minor genetic groups (genotypes I/1a, II/1b, III/2a, IV/2b, 2c, V/3a, 4a–4d, 4e, and 6a). It is noteworthy that we observed a major division in genetic distance between HCV isolates of genotype 2 and those of the other genotypes in the phylogenetic analyses of both gene sequences. Furthermore, the divergence of the minor genotypes within genotype 2 exhibits a degree of heterogeneity that is equivalent to that observed among the major genotypes. Analysis of the C gene from isolates Z5 and Z8, which had a unique 5' NC sequence (6) but from which we could not amplify the E1 gene, revealed that these isolates represented two additional genotypes. We

are provisionally assigning designations 4e and 4f to these genotypes that have not been described previously. Although Simmonds *et al.* (17) have published partial C gene sequences (i.e., nt 29–269) of HCV isolates that appear to be most closely related to our isolates of major genotype 4, final classification of these isolates must await completion of the gene sequence. Unfortunately, a sequence motif within the C gene (i.e., nt 186–221) that has been suggested to be predictive of genotype (8) does not reflect the genotype divisions observed by our analysis of the complete C gene. Overall, we have demonstrated that the genetic relatedness of HCV isolates is equivalent when analyzing the most conserved gene (C) and one of the most variable genes (E1) of the HCV

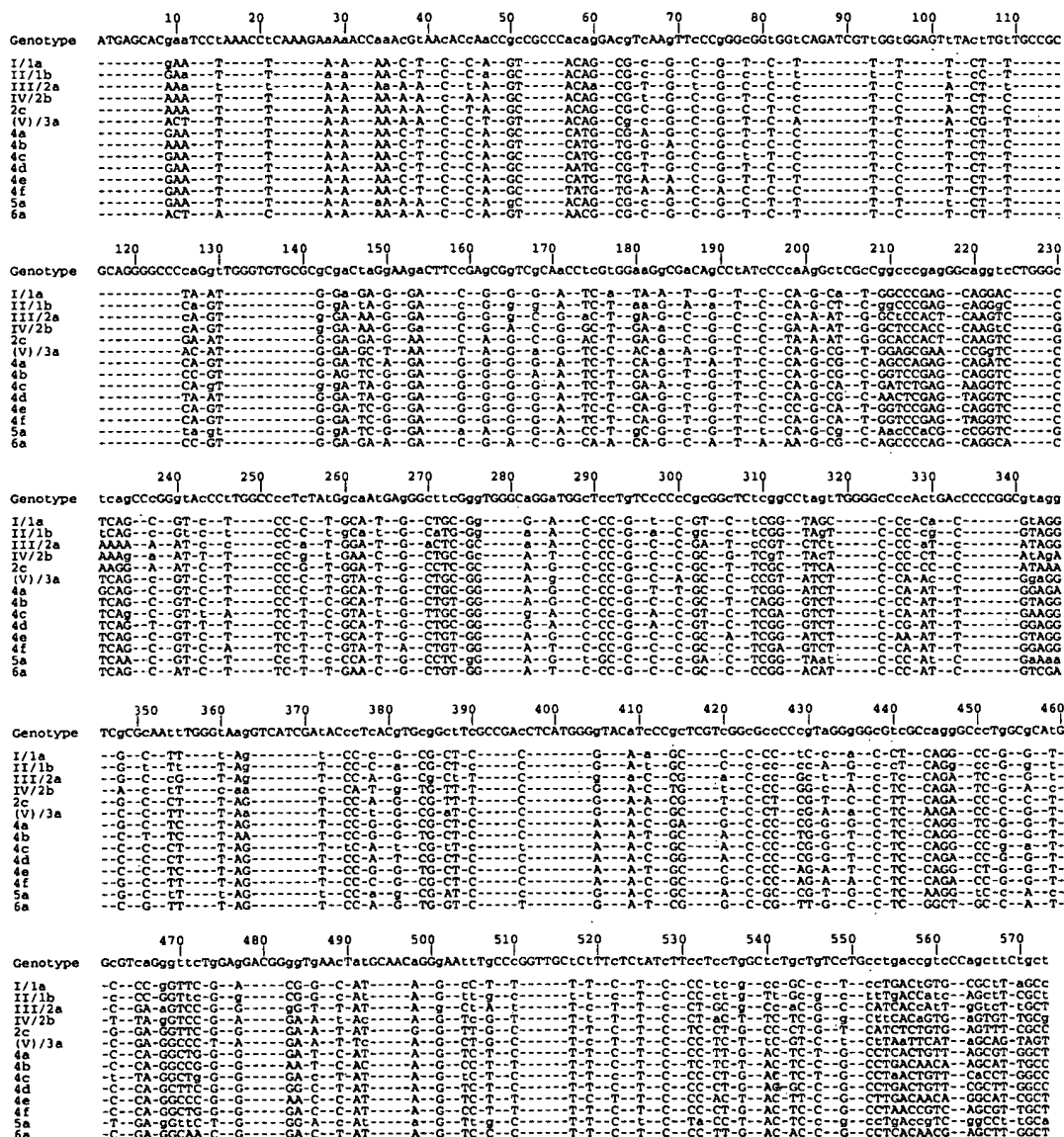


Fig. 2. Alignment of the consensus sequence of the C gene of the different genotypes of HCV. Consensus sequence of the C gene from all 52 HCV isolates studied is shown at the top. Furthermore, a consensus sequence of the C gene was obtained for genotypes I/1a, II/1b, III/2a, IV/2b, 3a, and 5a. The sequence of genotype 4c is represented by isolate Z6. Genotypes 4a, 4b, 4d, 4e, 4f, and 6a each contained only a single isolate. The exact HCV isolates representing the different genotypes can be seen in the phylogenetic tree of the C gene sequences in Fig. 1. Invariant nucleotides within a consensus sequence are capitalized and variable nucleotides are shown in lowercase letters. However, nucleotides that were invariant among all 52 HCV isolates are shown as dashes in the alignment. In the 14 nt positions where no consensus sequence was obtained, we show the nucleotide that differed from that of the other genotypes.

genome, providing strong evidence for the suggested division into major and minor genotypes.

To study further the heterogeneity of the C gene, we obtained the consensus sequence of this gene from the 52 HCV isolates (Fig. 2). We found that a total of 335 (58.5%) of the 573 nucleotides of the C gene were invariant among these HCV isolates. Nucleotides at the first and second codon positions were invariant at 70.7% and 81.7% of these positions, respectively, while nucleotides at the third position were invariant at only 23.0% of such positions. Stretches of 6 or more invariant nt were observed from nt 1–8, 22–27, 85–92, 110–125, 131–141, 334–340, 364–371, 397–404, and 511–516 and may be suitable for anchoring primers for amplification of HCV RNA in cDNA PCR assays. Finally, we documented the genotype-specific sequences within the C gene by aligning the consensus sequences of all 14 genotypes (Fig. 2). Although the full-length sequence of the C gene of isolates representing genotypes I/1a, II/1b, III/2a, IV/2b, and V/3a have been reported by others (2–5, 11), those of 9 of the 14 genotypes (i.e., 2c, 4a–4f, 5a, and 6a) have not been reported previously. Overall, we have mapped universally conserved sequences as well as genotype-specific sequences of the C gene among 14 genotypes of HCV.

Analysis of the Deduced Amino Acid Sequence of the C Gene. To study the heterogeneity of the C protein, we performed a multiple sequence alignment of the predicted amino acids for all 52 HCV isolates (data not shown) and obtained a consensus sequence (Fig. 3). The identities of the predicted 191 aa of the C protein among these HCV isolates were in the range 85.3–100.0%. A total of 132 (69.1%) of the 191 aa of the C protein were invariant. The most prevalent amino acids in the consensus sequence were glycine (13.6%), arginine (12.6%), proline (11.0%), and leucine (9.9%). The most conserved amino acids were tryptophan (5 of 5 aa invariant), aspartic acid (5 of 5 aa invariant), proline (19 of 21 aa invariant), and glycine (23 of 26 aa invariant). Previous analyses indicated that HCV is evolutionarily related to pestiviruses (15). In this regard, it is of interest to note that the C proteins of both viruses have a high content of proline residues (18), which are likely to be important in maintaining the structure of this protein. As is characteristic for a protein that binds to nucleic acid, we found that the C protein has conserved amino acids that are basic and positively charged, and these are capable of neutralizing the negative charge of the HCV RNA encapsidated by this protein (19). Specifically, >16% of the amino acids in the consensus sequence of the C protein of HCV are arginine and lysine that are located primarily in three clusters (i.e., from aa 6–23, 39–74, and 101–121) (20) (Fig. 3). The 10 arginine and lysine residues within aa 39–62 are invariant among all 52 HCV isolates, suggesting that this domain may

represent an important RNA-binding site. The capsid proteins of the related flavi- and pestiviruses (15) also have a high content of arginine and lysine (18, 19). Although there are three major hydrophilic regions (i.e., aa 2–23, 39–74, and 101–121) that are conserved in all 52 HCV isolates, the remainder of the C protein is hydrophobic. Interestingly, one such highly conserved hydrophobic domain at aa 24–39 is flanked by proline residues. The hydrophobic domains are likely to be involved in protein–protein and/or protein–RNA interactions during assembly of the nucleocapsid as well as in interaction with the lipoprotein envelope, as has been suggested for flaviviruses (19). Other significant observations are (i) a cluster of 5 invariant tryptophan residues at aa 76–107; (ii) the lack of an N-linked glycosylation site (NXT/S); (iii) two potential nuclear localization signals (i.e., PRRGPR at aa 38–43 and PRGRRQP at aa 58–64) that are present in all 52 HCV isolates (20); and (iv) a putative DNA-binding motif SPRG at aa 99–102, found in 51 of the 52 HCV isolates, with SP present in all 52 isolates. Our finding of conserved nuclear localization signals and a DNA-binding motif adds support to the hypothesis that the C protein of HCV might also function as a gene-regulatory protein (20). Furthermore, it has been suggested that the HCV C protein is posttranslationally modified through phosphorylation (20, 21). Interestingly, we found that the C protein of all 52 HCV isolates contained a SP motif that was recently demonstrated to be essential for C protein phosphorylation in hepadnaviruses (22). Our study demonstrates that the C protein has features that are highly conserved among the various genotypes of HCV and that are known to be characteristic of capsid proteins of other related viruses.

To study the heterogeneity of the C protein of different genotypes, we obtained the consensus sequence of the protein for all isolates comprising the 14 HCV genotypes (Fig. 3). We mapped the genotype-specific sequences within the C protein by then aligning these consensus sequences (Fig. 3). It should be noted that phylogenetic analysis of the amino acid sequence of the C proteins was not capable of resolving the minor groups within genotypes 1 and 4 because of the conservation of this protein (data not shown). Overall, we identified only a few type-specific amino acids (Fig. 3). One striking example was that isolates of genotype 4 have an additional methionine at position 20 that is specific for this major genetic group. Finally, we analyzed the conservation of the sequences surrounding the cleavage site between the C and the E1 proteins of the different genotypes, which has been determined to be between aa 191 (alanine) and 192 (tyrosine) in HCV isolates of genotype 1 (1). We previously found that the N-terminal amino acids of E1 were variable even within genotype 1 (7). In this study, we find that the

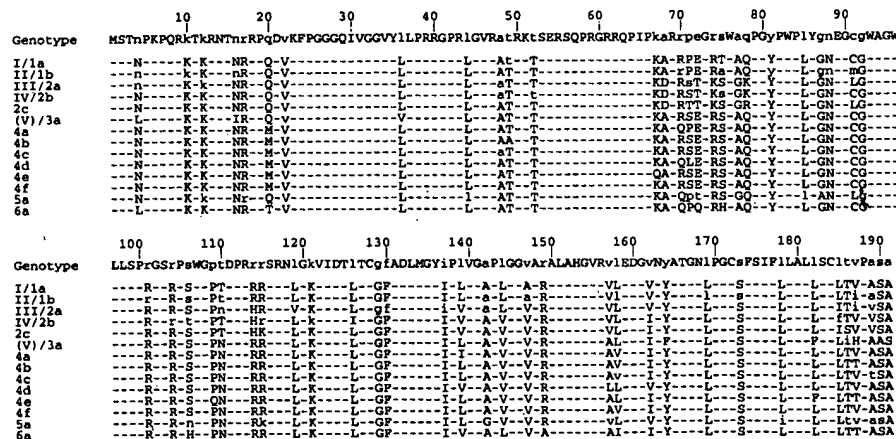


FIG. 3. Alignment of consensus sequence of deduced amino acid sequences of the C gene of the different genotypes of HCV. Consensus sequence of the C protein from all 52 HCV isolates studied is shown at the top. In the 2 aa positions where no consensus sequence was obtained, we show the amino acid that differed from that of other genotypes. See also legend to Fig. 2.

C-terminal sequence of C is SA in all but 1 of the 48 HCV isolates comprising genotypes 1, 2, 4, 5, and 6. However, all 4 HCV isolates of genotype 3 in this study, as well as isolates of genotype 3 published previously (11, 12), contain AS at this position. Thus, studies will be needed to determine the C/E1 cleavage site in genotype 3 isolates. Overall, we have mapped universally conserved sequences, as well as genotype-specific sequences, of the C protein among 14 genotypes of HCV.

Detection of antibodies directed against the HCV core protein is important in diagnosis of HCV infection. The recombinant C22-3 protein, spanning aa 2–120 of the C gene, is a major component of the commercially available second-generation anti-HCV tests. Several studies have indicated that the three major hydrophilic regions of the C protein contain linear immunogenic epitopes (summarized in ref. 23). For example, antibodies against synthetic peptides from aa 1–18, 51–68, and 101–118 were detected in infected patients (23). Our study demonstrates that, while these immunogenic regions are highly conserved, genotype-specific differences are observed at several amino acid positions that may influence the specificity and sensitivity of the serological tests (Fig. 3). One such example is that a single substitution at aa 110 has been demonstrated to affect seroreactivity (23). Despite the high degree of conservation in the immunodominant regions of the C protein among the different genotypes, it is possible that genetic heterogeneity of the C protein could lead to false-negative results in current serological tests.

Methods for Genotype Analysis. Several methods have been used to determine the genotype of HCV isolates without resorting to sequence analysis. These include PCR followed by (i) amplification with type-specific primers (24); (ii) determination of restriction-length polymorphism (17); and (iii) specific hybridization (25). The proposed methods have primarily been based on 5' NC and C sequences. Our previous studies suggested that 5' NC-based genotyping systems would be predictive of only the major genetic groups of HCV (6, 7). The most widely used C-based genotype system has been the PCR assay with type-specific primers that was designed for distinguishing HCV isolates of genotypes I/1a, II/1b, III/2a, IV/2b, and V/3a (11, 24). Since this system was developed before identification of genotypes 2c, 4a–4f, 5a, and 6a, there are significant limitations to this typing system. For example, the primers specific for genotype IV/2b (nt 270–251) are as highly conserved within our isolates of genotypes 4c and 6a as within the isolates of genotype IV/2b. Thus, this assay probably cannot distinguish among these genotypes. Another C-based approach involves distinguishing between genotypes 1 and 2 by type-specific antibody responses (26). Synthetic peptides composed of aa 65–81 were found to be genotype-specific for genotypes 1 and 2 in ELISAs. Our analysis of amino acid sequences demonstrated significant variation within isolates of genotypes 1 and 2. Thus, it is likely that these peptides will not identify all isolates of genotypes 1 and 2. Furthermore, the peptide for genotype 1 was highly conserved within isolates of genotypes 3 and 4 (Fig. 3) and might detect antibodies against these genotypes as well. It should be pointed out that most isolates of genotypes 3 and 4 had an identical amino acid sequence at positions 65–81. Overall, the proposed C-based genotyping systems should be revised in light of the C gene sequence data presented here, and a more definitive approach such as sequence analysis of gene regions that are predictive of genotype may be necessary for a definitive determination.

Conclusion. The genetic relatedness of HCV isolates is equivalent when analyzing the most conserved (i.e., C) and the most variable (i.e., E1) genes. The results of this study have implications for the taxonomy of HCV and for the diagnosis, prevention, and therapy of HCV infections.

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EXHIBIT 1

87/89

FIGURE 7J

Deleyes Peptide II

SEQ ID NO:	Genotype	
155-206	Cons.	1
155-176	type 1	MSTNPKPQRTKRTNTRRPQDVKFPGGQIVGGVYLLPRRPRPLGVKATKTSERSQPRGRROPIPKARIPeGraWaqPG/PWPIYgnEG-GNAGW
177-186	type 2	MSTNPKPQRTKRTNTRRPQDVKFPGGQIVGGVYLLPRRPRPLGVKATKTSERSQPRGRROPIPKARIPeGraWaqPG/PWPIYgnEG-GNAGW
187-190	type 3	MSTNPKPQRTKRTNTRRPQDVKFPGGQIVGGVYLLPRRPRPLGVKATKTSERSQPRGRROPIPKARIPeGraWaqPG/PWPIYgnEG-GNAGW
191-197	type 4	MSTNPKPQRTKRTNTRRPQDVKFPGGQIVGGVYLLPRRPRPLGVKATKTSERSQPRGRROPIPKARIPeGraWaqPG/PWPIYgnEG-GNAGW
198-205	type 5	MSTNPKPQRTKRTNTRRPQDVKFPGGQIVGGVYLLPRRPRPLGVKATKTSERSQPRGRROPIPKARIPeGraWaqPG/PWPIYgnEG-GNAGW
198-206	type 6	MSTNPKPQRTKRTNTRRPQDVKFPGGQIVGGVYLLPRRPRPLGVKATKTSERSQPRGRROPIPKARIPeGraWaqPG/PWPIYgnEG-GNAGW
97	Cons.	97
155-206	Genotype	1
155-176	type 1	LLSPGSRPawGpCpPRrSRNlGKVIDTLTCGFADLNGYIPIVgaPIGGvARALAHGVRVLEDVNVATUNLPSCaFSIFILALISCLTVPaSa
177-186	type 2	LLSPGSRPawGpCpPRrSRNlGKVIDTLTCGFADLNGYIPIVgaPIGGvARALAHGVRVLEDVNVATUNLPSCaFSIFILALISCLTVPaSa
187-190	type 3	LLSPGSRPawGpCpPRrSRNlGKVIDTLTCGFADLNGYIPIVgaPIGGvARALAHGVRVLEDVNVATUNLPSCaFSIFILALISCLTVPaSa
191-197	type 4	LLSPGSRPawGpCpPRrSRNlGKVIDTLTCGFADLNGYIPIVgaPIGGvARALAHGVRVLEDVNVATUNLPSCaFSIFILALISCLTVPaSa
198-205	type 5	LLSPGSRPawGpCpPRrSRNlGKVIDTLTCGFADLNGYIPIVgaPIGGvARALAHGVRVLEDVNVATUNLPSCaFSIFILALISCLTVPaSa
198-206	type 6	LLSPGSRPawGpCpPRrSRNlGKVIDTLTCGFADLNGYIPIVgaPIGGvARALAHGVRVLEDVNVATUNLPSCaFSIFILALISCLTVPaSa

EXHIBIT 2

87/89

FIGURE 7J

Shirai C7-P106

SEQ ID NO:	Genotype	cons.	1
155-206	cons.		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW
155-176	type 1		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW
177-186	type 2		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW
187-190	type 3		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW
191-197	type 4		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW
198-205	type 5		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW
198-206	type 6		MSTNPKPQKTKRNTNRPQDVKFPGGQIVGGVYLLPRRPRRLGVRATKTSERSQPRGRQPIPKARpeGrawaQPG/PWPLYgnEGGwAGW

SEQ ID NO:	Genotype	cons.	97
155-206	cons.		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa
155-176	type 1		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa
177-186	type 2		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa
187-190	type 3		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa
191-197	type 4		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa
198-205	type 5		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa
198-206	type 6		LLSPfGSrPwngPtdPRrrSRNIGKVIDTITCGfNDLNGYIHLVgaPlGGvArAaLHGVRVLEDGvNyATGNlPScfSIFlAlLSCLtvpAsa

EXHIBIT 3

87/89

FIGURE 73

G15V R15P

Ferroni

SEQ ID NO:	Genotype	Cons.	1
155-206	type 1		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
155-176	type 2		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
177-186	type 3		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
187-190	type 4		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
191-197	type 5		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
198-205	type 6		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
198-206	type 6		MSTNPKPQKTKRNTNRPPQVVKFPGGGQIVGGVYLLPRRGPRIGVRATKTSERSQPRGRQPIPKARIPeGrswaQPG/PWPLYgnEG-P9WAGW
SEQ ID NO:	Genotype	Cons.	97
155-206	type 1		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa
155-176	type 2		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa
177-186	type 3		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa
187-190	type 4		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa
191-197	type 5		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa
198-205	type 6		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa
198-206	type 6		LLSPGSRPwngpdprrrrsrnlgaviddlttcgfdlmgvplvgaPlGGvAALAHGVRVJEDSVRVATGNIPeCsfSIFILALISCLtvpAsa